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13. ABSTRACT (Maximum 200 Words)

Germline mutations of BRCA1 confer an increased risk for breast and ovarian cancer in women and prostate cancer in men. Recent studies suggest that the tumor suppressor activity of BRCA1 is due, in part, to physical/functional interactions with other tumor suppressors, including p53 and the retinoblastoma (RB) protein. Two RB binding sites on BRCA1 were identified, one in the C-terminal BRCT domain and one in the N-terminus, between aa 304 and 394 (Yarden and Brody, *PNAS USA* 96: 4983-4988, 1999; Aprelikova et al. *PNAS USA* 96: 11866-11871, 1999). The N-terminal region of BRCA1 contains a consensus RB binding motif (^{358}LXCXE), but the role of this site in mediating RB binding and BRCA1/RB functional activity is unknown. Our studies indicate that the BRCA1 interacts with RB, through a binding site between aa 302 and 440, but the binding is <u>not</u> dependent on the LXCXE motif. Nor does the interaction require an intact A/B binding pocket of RB. Transient or stable expression of a wild-type BRCA1 gene (wtBRCA1) caused down-regulation of expression of RB, p107 and p130, associated with a chemosensitivity to DNA-damaging agents. In contrast, expression of an LXCXE-defective BRCA1 mutant (LXCXE → RXRXH) did not cause down-regulation of the RB proteins and the induction of chemoresistance. Our findings suggest that some biologic functions of BRCA1 (eg., chemosensitization) are due, in part, to down-regulation of RB family proteins mediated by an LXCXE site embedded within the N-terminal RB binding site.

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INTRODUCTION

BRCA1. Mutations of BRCA1 (17q21) confer increased risk for breast, ovarian, and prostatic cancers (1-5). Within BRCA1 early-onset breast cancer families, the ratio of ovarian/breast cancers is high for 5' and low for 3' mutations, suggesting possible cell type-specific tumor suppressor activity of the N and C termini of BRCA1 (6). The BRCA1 gene encodes an 1863 amino acid (aa) nuclear phosphoprotein with an N-terminal RING and a C-terminal transcriptional activation domain (TAD) (1,7,8) (Fig. 1). The BRCA1 RING domain mediates interactions with cyclins, cyclin-dependent kinases (CDKs), E2F proteins, a novel C-terminal ubiquitin hydrolase (BAP1), and a novel RING protein (BARD1) (9-12); while the C-terminal minimal TAD interacts with the RNA polymerase II holoenzyme (13), possibly via binding to RNA helicase A (95). BRCA1 also contains both a classic (LXCXE, aa 358-362) (14) and an atypical (LXCXXE, aa 440-445) (15) consensus RB family protein binding motif; but it is not known if and how these putative RB protein binding motifs function in mediating tumor suppression. Unselected invasive breast cancers exhibited decreased BRCA1 mRNA expression (17) and a loss of BRCA1 immunochemical staining (90) compared to non-invasive cancers and benign tissue (17), suggesting a role for BRCA1 in suppressing sporadic breast cancers. While BRCA1 expression suppressed and antisense inhibition of BRCA1 stimulated the growth of adult human breast and ovarian cancers (16,17), BRCA1 may be essential for embryo cell proliferation, since Brca1 (-/-) mice died during early embryogenesis due to a severe defect in cell proliferation (30). Thus, BRCA1 appears to negatively and positively regulate cell proliferation in different contexts.

BRCA1 was implicated in regulation of breast and ovarian cancer cell growth (16,17), cell cycle progression (18-21), apoptosis (22-24), DNA repair (24-28), and maintenance of genomic integrity (29). The mechanisms of these activities are not well understood, but recent studies provide clues. Thus, BRCA1 associates with Rad51, a mammalian DNA recombinase, *in vivo* (25). After DNA damage, both proteins translocate to DNA repair sites (26), implicating BRCA1 in Rad51 pathways of DNA recombination. Brca1 (-/-) mouse fibroblasts are defective in transcription-coupled repair of DNA damage from ionizing radiation (28). We showed that BRCA1 and BRCA2 expression are co-ordinately down-regulated after certain forms of DNA damage (56,57, see APPENDIX). We also showed that unregulated BRCA1 expression confers chemosensitivity, susceptibility to apoptosis, and reduced DNA repair activity in prostate (24) and breast cancer (see preliminary studies). Interestingly, BRCA2 directly interacts with Rad51 (63); and several studies implicate BRCA2 in repair of double-stranded DNA breaks (64,65). This observation may fit with our finding that BRCA2 expression is up-regulated in cells transfected with BRCA1 (24).

BRCA1 associates with the C-terminus of p53 via a region mapping to aa 224-500 of BRCA1 and enhances transcription of p53 target genes, including cell cycle inhibitor p21$^{WAF1/CIP1}$ and apoptosis gene Bax (91,92). BRCA1 associates with transcriptional co-activator CBP (93); and we showed that BRCA1 down-regulates expression of Bcl-2 and p300 (a homolog of CBP), two proteins that mediate chemoresistance (24). BRCA1 associates with a protein kinase via a region mapping to aa 329-435; and deletion of this region abolishes growth suppression by BRCA1 (94). The sites of BRCA1 involved in interaction with p53 and BRCA1-associated protein kinase include ^{358}LXCXE. The C-terminal TAD of BRCA1 contains BRCT sequences, a motif found in 40-50 proteins involved in the DNA damage response (97). However, BRCT may not be the only site involved in the DNA damage response, since preliminary studies show that expression of a BRCA1 gene with an LXCXE site mutation confers chemoresistance. The role of LXCXE, LXCXXE, and BRCA1:RB interactions in mediating BRCA1 function is the subject of this proposal.

Rb gene family. The Rb1 gene (13q14) plays major roles in regulation of cell cycle progression, differentiation, and apoptosis. The activated (hypo-phosphorylated) RB1 protein (p105) inhibits cell cycle

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progression from G1 → S, in part, via an interaction between the large A/B pocket of RB1 and the activation domains of E2F family transcription factors, resulting in repression of E2F target genes (reviwed in 31). The cell cycle inhibitory activity of RB1 is regulated via interactions of the standard A/B binding pocket domain of RB1 with the LXCXE motif of target proteins. For example, interactions between RB1 and cell cycle regulatory proteins (G1/S cyclins and CDKs) and viral oncoproteins (SV40 large T antigen, adenovirus E1A, human papillomavirus E7) inactivate the cell cycle inhibitory activity of RB1 (32-36); while binding of RB1 to an LXCXE-like motif (IXCXE) of histone deacetylase HDAC1 recruits HDAC1 to E2F1 target promoters and mediates transcriptional repression by RB1 (37,38).

The A/B and C domains are conserved in Rb1 gene family proteins p107 and p130, which also bind to LXCXE and regulate cell cycle-dependent transcription (14,39-41). Activities of p107 and p130 overlap with but are not identical to RB1; and these proteins may partially substitute for RB1 functions. The standard A/B binding pocket, which regulates the phosphorylation state and cell cycle regulatory activity of RB1, is the site of most tumor-associated Rb1 mutations (31). However, accumulating evidence suggests the existence of distinct classes of Rb1 mutations associated with low vs high penetrance tumor phenotypes (42-44). The existence of mutants that confer high vs low probability of tumor development suggest that some mutant RB1 proteins retain partial wild-type tumor suppressor activity; and several recent studies provide experimental verification of this idea (42,43). It is tempting to speculate that these mutants differ in the capacity for structural or functional interaction with BRCA1.

Significance. BRCA1 functions in growth control, apoptosis, and DNA damage pathways, but the mechanisms of these functions are unclear. RB1 restricts progression from G1 → S by blocking transcription of genes needed for DNA synthesis, via complex protein interactions. Interaction of the A/B pocket of RB family proteins (RB1, p107, p130) and LXCXE or IXCXE motifs of cell proteins (cyclins, CDKs, HDAC1) modulates transcriptional repression by RB. BRCA1 has typical (LXCXE) and atypical (LXCXXE) RB binding motifs, but the physiologic importance of BRCA1:RB interactions is unknown. Preliminary studies suggest that: 1) expression of mutant BRCA1 defective in the RB binding motif in prostate and breast cancer cells confers an altered phenotype, characterized by increased growth rate, chemoresistance, and resistance to apoptosis; and 2) BRCA1 and RB1 interact *in vivo* and *in vitro*. An LXCXE-mutant BRCA1 differentially suppressed *in vivo* tumor growth in cells with wild-type RB1 (MCF-7) vs mutant RB1 (DU-145), suggesting a role for the LXCXE site and BRCA1:RB1 interaction in tumor suppression. This proposal will test the hypothesis that BRCA1:RB interactions mediate breast cancer suppression. Because RB1 molecular pathways have been dissected in depth, these studies will open a new avenue of research on the role of BRCA1 in molecular carcinogenesis. Thus, it would not be surprising to find that certain BRCA1 mutations are carcinogenic because they disrupt the function of RB1 and circumvent the need for an Rb1 mutation to enable breast cancer growth. We believe that BRCA1 and RB1 collaborate in restricting proliferation and in signalling DNA damage and/or executing an apoptosis program in genetically damaged cells. Knowledge obtained from these studies may lead to novel genetic strategies for breast cancer prevention or treatment.

BODY

The proposed studies which have been completed are summarized as following and in the Manuscript attached in Appendices:

SA1. Effect of disruption of BRCA1:RB interaction on human breast cancer (HBC) cell phenotype.

In SA1, we will: a) confirm and extend preliminary studies by determining how BRCA1-RXRXH alters the phenotype of HBC cells with wild-type vs mutant Rb1; b) determine if these alterations are directly linked to LXCXE by demonstrating similar alterations in cells expressing a BRCA1 gene with a different LXCXE mutation; and c) assess the role of an atypical RB binding motif of BRCA1 (LXCXXE) in modulating HBC phenotype.

SA1-a. Phenotype of HBC cell lines transfected with BRCA1-RXRXH.
1. Isolation of BRCA1-RXRXH, wtBRCA1, and control HBC cell clones.

We have successfully established T47 and MCF-7, two breast cancer cell lines, with stable-transfection of pcBRCA1-385 (=wtBRCA1), pc-mutBRCA1-RXRXH (=BRCA1-RXRXH), and empty pcDNA3 vector (=neo) through selected in G418 as described before (24 and The Manuscript see Appendices).   To confirm transgene expression, we used MS110 (Ab-1, Oncogene Research Products), a monoclonal against the N-terminus of BRCA1, that detects both wtBRCA1 and BRCA1-RXRXH on Western blots (SEE Fig. 7, MS in APPENDICES).   We also confirmed wtBRCA1 and mutant BRCA1 mRNA expression by semi-quantitative RT-PCR, as described before by us (24,56,57).

2. Phenotypic characteristics of LXCXE mutant BRCA1 vs wtBRCA1 vs control HBC cell clones.

Rationale.   After confirming transgene expression in BRCA1-transfected cell clones, we investigated and compared phenotypic characteristics in BRCA1-RXRXH vs wtBRCA1 vs control (neo) clones, including: *in vitro* growth and cell cycle kinetics, response to cytotoxic DNA-damaging agents, DNA repair capacity, and expression of key cell regulatory proteins that may modulate these processes.   For each cell line (MCF-7 cells and Du-145, one prostate cancer cell line that have been established in our preliminary studies),   three clones of each clonal were assayed; and each experiment was repeated at least twice.   Response parameters (eg., population doubling times, ED_{50}s for drug survival will be compared among the three clonal types.   Assays are briefly outlined below.

In vitro proliferation.   These studies will tell us if the putative BRCA1:RB interaction affects cell proliferation rates under conditions conducive to rapid growth or under stressful conditions (low serum, clonal density, lack of contact with substrate).   We found that BRCA1-RXRXH cells had slightly fast growth rate compared to control-NEO cells under normal growth condition (10% serum DMEM) (SEE Fig. 8a, MS in APPENDICES).   Growth rate will be further compared under under stressful conditions (low serum, clonal density, lack of contact with substrate) in coming year.

Cell cycle kinetics.   Both BRCA1 and RB1 function in cell cycle check-points, mechanisms that ensure orderly replication of the genome and nuclear/cytoplasmic division (61).   Failure of check-points may lead to cytogenetic alterations and/or to altered chemo/radiosensitivity, since different forms of damage are preferentially repaired in different cell cycle compartments.   Cell cycle distributions of asynchronously proliferating cells will be determined by flow cytometry of propidium iodide-stained nuclei (24) in coming year as described in proposal.   Cell cycle distributions will be calculated from the DNA histograms using the MODFIT program.   BRCA1 transgenes may confer cell cycle alterations only become obvious when the cells are perturbed.

Response to cytotoxic DNA-damaging agents

Choice of agents to be studied. BRCA1 may preferentially modulate the response to some agents, but not others, depending upon the particular agent's mechanism of action and type of DNA lesion(s) produced. Thus, we described alterations in BRCA1 and BRCA2 expression only in response to specific DNA-damaging agents (56,57); and Brca1 (-/-) murine fibroblasts exhibited a defect in transcription coupled repair of DNA damage induced by ionizing radiation but not UV (28). In SA1a, we investigated cellular response to adriamycin (ADR) and camptothecin (CPT), two DNA damaging agents in different transfection cells. Dose-responses for ADR were tested in MTT screening assays, a spectrophotometric assay based on mitochondrial conversion of a tetrazolium salt to formazan (45), over a dose range that yields cell viability values from < 10% to > 90%; and ED_{50}s iso-dose values (ie., dose of agent required to reduce cell viability to 50% of control) was calculated. Differences in viability of BRCA1-RXRXH vs wtBRCA1 vs control (neo) clones in MTT assays will be confirmed by colony formation (a measure of reproductive viability) and trypan blue dye exclusion [a measure of cell membrane integrity (56,59)] in coming year. We found that BRCA1-RXRXH cells exhibited a significantly resistant chemosensitivity to ADR and CPT compared to Control-NEO cells (SEE Fig. 7b and 7c, MS in APPENDICES).

Cell death may be due to apoptotic (genetically programmed) and/or non-apoptotic (cell necrosis) pathways. Inhibition of apoptosis-induction pathways may be key events for carcinogenesis, permitting survival of genetically altered cells, and for acquisition of chemo/radioresistance (62). Above studies indicate wtBRCA1 clones of DU-145 and MCF-7 are more susceptible than control (parental/neo) clones to apoptosis induction by ADR and CPT; while BRCA1-RXRXH clones are more resistant to apoptosis induction by the same agents. The striking difference in apoptosis in cell lines with unregulated expression of wtBRCA1 compared to the BRCA1-RXRXH mutant suggests a major role for the LXCXE site in activation and/or execution of an apoptosis pathway(s).

Apoptotic DNA was visualized on agarose gels (24,53). ADR and CPT that differentially alter cell viability in BRCA1 vs control transfected cells were tested over a range of agent doses, to allow comparisons at equal doses or equal cell survival. We found that wtBRCA1 clones were more susceptible and BRCA1-RXRXH clones less susceptible than controls to apoptosis induction, these results may reflect two possibilities: 1) the LXCXE site modulates the threshhold DNA damage level required for entry into apoptosis; and/or 2) LXCXE modulates the signalling or repair of DNA damage, resulting in an altered amount of damage signalled to the apoptosis machinery. These possibilities will be distinguished by examining the relationship between residual DNA lesions (see below) and extent of apoptosis. There comparison will be performed in coming year. These studies will focus on two agents (ADR and X-rays) that yield single-strand (SSBs) and double-strand (DSBs) breaks. We will quantitate breaks as a function of dose and time after treatment, by alkaline elution (SSBs) and neutral elution (DSBs), using non-proteinizing polycarbonate filters (58).

We also determined tumorigenesis of BRCA1-RXRXH cells in vivo compared with Control-NEO cells and wtBRCA1 cells and found that BRCA1-RXRXH mutation cells grew much fast than Control-NEO cells, wtBRCA1 cells had much slow growth rate in vivo (SEE Fig. 8b, MS in APPENDICES).

SA1-b. Phenotype of HBC cells transfected with BRCA1 containing another inactivating mutation of LXCXE.
Rationale. The BRCA1-RXRXH mutation presumably inactivates LXCXE-dependent BRCA1:RB interaction(s), but it is possible that this mutation causes other alterations of BRCA1 tertiary structure that cause changes in DNA-damage response unrelated to the LXCXE site. The finding of similar phenotypes in different BRCA1-RXRXH transfected HBC clones does not rule out this possibility, but the finding of a similar phenotype conferred by a different LXCXE mutation would provide more convincing evidence that

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the observed phenotype is directly related to disruption of the LXCXE site. The goal of SA1-b is to verify the importance of BRCA1:RB interaction by testing the phenotype of HBC cell clones transfected with BRCA1 containing another inactivating mutation of LXCXE.

Site-directed mutagenesis of BRCA1. We will use oligonucleotide-directed site-specific mutagenesis of wtBRCA1 expression plasmid pcBRCA1-385 to generate an expression plasmid for BRCA1 with LXCXE deleted (BRCA1 δ LXCXE), using the MORPH™ Site-Directed Plasmid DNA Mutagenesis Kit (5 Prime → 3 Prime) and oligoprimer #1 in coming year as proposed.

SA2. Protein:protein interactions between BRCA1 and RB family proteins.

Goals. In SA2, we will: a) confirm and extend preliminary studies suggesting *in vivo* association of BRCA1 and RB family proteins (RB1, p107, p130) in HBC cell lines; b) assess the roles of LXCXE, LXCXXE, and other sites in BRCA1:RB interactions; and c) establish the importance of these interactions for transcriptional regulation.

SA2-a. *In vivo* **interaction between BRCA1 and RB family pocket proteins (RB, p107, p130) in HBC cell lines. Rationale.** In SA2-a, we will extend preliminary studies to investigate the association of BRCA1 with different RB family proteins in HBC cells with wild-type vs mutant Rb1. We will to address two specific issues: 1) does BRCA1 associate with p107 and p130 in HBC cells?; and 2) does *in vivo* association of BRCA1 and RB1 require an intact A/B pocket? If BRCA1:RB1 association occurs only via LXCXE-like sites, then mutant RB1 proteins with defective A/B pockets should not associate with BRCA1. However, preliminary studies suggest otherwise, since DU-145 mutant RB1 associates with BRCA1 *in vivo* and BRCA1-RXRXH appears to bind RB1 *in vitro*.

BRCA1:RB family protein association by immunoprecipitation (IP) assay. We have assessed BRCA1:RB association by IP of cells with wt vs mutant Rb1 genes. To optimize chances of detecting an interaction, we used low stringency IP conditions and pre-label cell proteins with ^{35}S-methionine to allow sensitive autoradiographic detection (71,72). This procedure requires a second IP (BRCA1 IP → RB1/p107/p130 IP) to verify the identity of proteins of expected M_r, but has the added benefit of allowing detection of other proteins in the BRCA1 immunocomplex. Clues to the identity of these proteins are obtained from the M_r of bands precipitated in stoichiometric quantities along with BRCA1 and RB. The presence of a suspected protein can be confirmed by another IP using an antibody (Ab) specific for that protein. Controls included: 1) pre-incubation of IP Ab ± block [immunizing peptide or *in vitro* translated protein (see below)]; 2) use of another primary Ab for IP; and 3) IP with control Ab (normal mouse IgG or irrelevant Ab). In MCF-7 and Du-145 cells, we have adminstrated physical interaction of BRCA1 and RB1 in vivo. BRCA1-RXRXH did not affect bindings of BRCA1 to RB1., suggesting that an *in vivo* BRCA1:RB1 interaction does not involve LXCXE or the A/B pocket (SEE Fig. 3, MS in APPENDUCES)

SA2-b. **Role of LXCXE, LXCXXE, and other sites in mediating BRCA1:RB family protein interactions.**

The goals of SA2-b are to: 1) identify each of the binding sites for RB1 on the BRCA1 protein; 2) determine if p107 and/or p130 can also bind to these sites; and 3) for each RB1 binding site, determine if the BRCA1:RB1 interaction involves the A/B binding pocket domain as opposed to a different domain of RB1.

Assay of in vitro BRCA1:RB interactions by GST capture

RB1 binding sites on BRCA1. To identify RB1 binding sites, we used GST pull-down assays (34) to examine binding of IVT ^{35}S-methionine labelled BRCA1 proteins to beads coated with GST-RB1 fusion protein. This strategy allowed us to rapidly screen BRCA1 mutants for binding to wt-RB1, since: 1) IVT mutant and wtBRCA1 can be prepared directly from plasmid pcDNA3, using the T7 promoter; and 2) we

have GST-Rb1 expression plasmids for wt-Rb1 (pGEX-wtRb1) and two A/B pocket mutants (pGEX-Rb1 δ Ex21 and pGEX-Rb1 δ Ex22) cloned into pGEX2T1. First, we tested beads coated with GST-RB1 vs GST alone (control) for pull-down of IVT BRCA1 proteins, to establish overall structural requirements (LXCXE plus non-LXCXE dependent) for binding to RB1.

The mutant BRCA1 expression plasmids currently available for testing are illustrated in Fig. 1 (SEE MS in APPENDICES). Additional mutant BRCA1 expression plasmids from SA1 will also be examined for RB1 binding: BRCA1 δ LXCXE, RXRXXH, δ LXCXXE, (RXRXH+RXRXXH), and δ (LXCXE+LXCXXE). As suggested by our studies(SEE Fig 2, MS in APPENDICES), these assays indicated the presence of a second non-LXCXE like binding site on BRCA1, we localized this site by testing a series of C-terminal deletion mutants of BRCA1-RXRXH for pull-down by wild-type and A/B pocket deleted forms of GST-RB1. See detail discussion in MS (APPENDICES)

Binding of p107 and p130 to BRCA1. It is possible that the context (surrounding amino acids) of the LXCXE and LXCXXE sites determine the binding specificity among different RB family members to BRCA1. We utilized GST pull-down assays and found that GST-p107 and GST-p130 could pull down IVT wtBRCA1 (SEE Fig. 2e, MS in APPENDICES). p107 or p130 also associate with BRCA1. If so, we will identify the binding site(s) on BRCA1.

SA2-C.
These studies are carried out or will be examined. This aim will be studied in coming year. Our fist year work has been summarized and submitted to Molecular Biology of the Cell for publication, the detail discussion on our findings is given in our manuscript (SEE APPENDICES).

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Reportable Outcomes

Abstract:

Saijun Fan, Jin Bo Xiong, Yong Xian Ma, Ren-qi Yuan, Qinghui Meng, Itzhak D. Goldberg, Eliot M. Rosen Function Role of the BRCA1 LXCXE Motif in Regulation of RB Family Protein Expression and Cellular Chemosensitivity, but not RB Protein Binding. Submitted to *The 92nd Annual Meeting of American Association of Cancer Research*, New Orleans, March, 24-28, 2000.

Manuscript:

Saijun Fan, Jin Bo Xiong, Yong Xian Ma, Ren-qi Yuan, Qinghui Meng, Itzhak D. Goldberg, Eliot M. Rosen Function Role of the BRCA1 LXCXE Motif in Regulation of RB Family Protein Expression and Cellular Chemosensitivity, but not RB Protein Binding. *Molecular Biology of the Cell*, under review, 2000.

CONCLUSION

our findings suggest that the LXCXE motif within the N-terminal RB-binding domain of BRCA1 is not necessary for the physical BRCA1:RB interaction but is required for two potential functional consequences of that interaction: 1) the down-regulation of expression of RB family proteins; and 2) the BRCA1-induced chemosensitivity and susceptibility to apoptosis induction by DNA-damaging agents.

Thus, the [358]LXCXE motif of BRCA1 is a functionally important site in mediating some of the tumor suppressor functions of BRCA1 and RB.

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APPENDICES

Concept Proposal Submission Form

This form has been set to accept 12 point Times New Roman font. For symbols use either (1) the Windows Character Map (under Accessories) or (2) from Word in a separate document, select Insert-Symbol...-(normal text). The use of other fonts is not recommended since the information may not be accurately transmitted.

☒ By checking this box, I attest that I (1) have a masters or doctoral degree (e.g., M.S., M.A., Ph.D., D.Sc., D.N.S., M.D., D.O., etc.) from an accredited institution; (2) have access to the necessary space and equipment to perform the proposed studies; and (3) am employed by an eligible institution (i.e., for-profit and nonprofit organizations, public and private, such as universities, colleges, hospitals, laboratories, companies, and agencies of local, state, and federal governments, including military laboratories). Therefore, I am eligible to submit this proposal.

1. Proposal Title (160 character limit): BRCA1:RB Interaction in Breast Cancer Suppression

2. Principal Investigator (PI):

    Last Name Fan First Name Saijun MI

3. Contact Information for PI:

    Organization Name Long Island Jewish Medical Center

    Department Name (if none, leave blank) Radiation Oncology

    Street Address Line 1 270-005, 76th Street

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    City New HydePark State NY Country USA Zip Code 11040

    Phone 718-470-7456 Fax 718-470-9756

    E-mail fan@lij.edu

4. Administrative Representative Authorized to Conduct Negotiations:

    Last Name Altman First Name Jacki MI

5. Contact Information for Administrative Representative Authorized to Conduct Negotiations:

Organization Name <u>Long Island Jewish Medical Center</u>

Department Name (if none, leave blank) <u>Office of Grants and Contracts</u>

Street Address Line 1 <u>5 Dakota Drive</u>

Street Address Line 2 (if no line 2, leave blank)

City <u>New York</u> State <u>NY</u> Country _____ Zip Code <u>11042</u>

Phone <u>615-719-3100</u> Fax <u>516-719-311</u>

E-mail <u>jacki@nshs.edu</u>

6. Content Area of the Proposal
Please indicate up to two areas of emphasis to best describe your proposal. Enter the appropriate codes in the drop-down fields below.

01	Behavioral and Psychosocial Sciences	09	Health Care Delivery
02	Cell Biology	10	Immunology
03	Clinical and Experimental Therapeutics	11	Pathobiology
04	Complementary and Alternative Medicine	12	Primary Prevention
05	Detection and Diagnosis	13	Radiation Sciences
06	Endocrinology	14	Research Resources
07	Epidemiology	15	Other, specify
08	Genetics and Molecular Biology		

Primary Proposal Content Area: 08   Secondary Proposal Content Area (Optional):

7. Will animals be used in the proposed work?   No

8. Will human subjects be used in the proposed work?   No

9. Will human anatomical substances, including primary and established
cell lines, be used in the proposed work?   No

10. Are laboratory experiments planned?   Yes

11. PI gender (optional, select one):   Male

12. PI ethnicity (optional, select one):   Asian

If "Other" selected, specify

Data collected for questions 11 and 12 will be reported outside the Department of Defense only

as grouped data without personal identifiers. Disclosure of this information is voluntary.

13. Budget Summary
Please adjust the indirect costs for your proposal below:

Direct Costs $50,000.00

Indirect Costs $0.00

Total Budget $50,000.00

14. Proposal Body
In the space below, please provide a clear and concise overview of the proposed work in 5,500 characters or less (~1 page). As appropriate, include the hypothesis, supporting rationale, objectives, relevance to breast cancer, and a general plan for how the project will be executed. Figures are not permitted and the use of tables is discouraged. Proposals will be reviewed by diverse panels of scientists, clinicians, and consumer advocates; therefore, applicants should consider the varied backgrounds of the reviewers when preparing proposals.

Inherited mutations of the BRCA1 gene (17q21) confer increased risk for breast, ovarian, and prostate cancers. BRCA1 has a consensus retinoblastoma (RB) family protein binding motif LXCXE (aa 358-362). Various studies implicate BRCA1 in regulation of cell proliferation, cell cycle progression, apoptosis, and DNA repair/recombination events; but the roles of the LXCXE motif and BRCA1:RB interactions in mediating these activities and cancer suppression are unknown. In preliminary studies, we found that expression of a BRCA1 gene with an inactivating mutation of LXCXE conferred an altered phenotype characterized by resistance to DNA-damaging agents and to apoptosis induction in both cell types and by differential in vivo tumor suppression in cells with wild-type RB1 (MCF-7) vs A/B pocket mutant RB1 (DU-145). We hypothesize that BRCA1 interactions with RB family proteins mediate human breast cancer (HBC) suppressor functions. To test this hypothesis, we will carry out two specific aims. First, we will stably express LXCXE-mutant, LXCXXE-mutant, double-mutant BRCA1, wild-type BRCA1 (positive control), and empty vector (negative control) in HBC cell lines with wild-type and mutant RB1; and we will compare clonal phenotypes with respect to proliferation, cell cycle kinetics, chemosensitivity, and DNA repair activity. Secondly, we will assess in vivo and in vitro interactions between BRCA1 and RB family proteins, determine the role of LXCXE, LXCXXE, and other sites in mediating these interactions, and assess the functional importance of these interactions. These studies will help to elucidate mechanisms of BRCA1 tumor suppression. The knowledge gained from these studies may provide an experimental basis for development of new genetic strategies for breast cancer control.

15. References
No references are required for these submissions. Up to 5 references pertinent to the proposed study may be included in the boxes below. Limit each reference to 225 characters (~3 lines).

1.

2.	
3.	
4.	
5.	

16. Principal Investigator Biographical Sketch

A biographical sketch of the PI must be submitted with the proposal but will not be considered in the peer or programmatic review process. A list of significant publications and a succinct summary of the investigator's professional experience in and/or potential for contribution to breast cancer research should be incorporated into the biographical sketch.

Biographical Sketch

Principal Investigator Name: Saijun Fan

Position Title: Assistant Professor

EDUCATION/TRAINING: (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.) Include the name of the institution and location, degree earned (if applicable), years attended, and field of study. Limit to 800 characters.

EDUCATION:	December 1989 - September 1992 (Ph.D)
	Department of Chemistry
	University of Leicester
	Leicester, United Kingdom
	September 1979 - July 1984 (BS, MD)
	Department of Radiation Biology
	Suchou Medical College
	Suchou, P. R. China

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with your present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List in chronological order the titles, all authors, and complete references for all publications during the past 3 years and earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds character limitations, select the most pertinent publications. Limit to 13,500 characters (~2.5 pages).

PROFESSIONAL EMPLOYMENT:

October 1996 - Now   Principal Investigator &
            Assistant Professor
            Department of Radiation Oncology
            Long Island Jewish Medical Center
            (The Campus for the Albert Einstein
            College of Medicine)
            New Hyde Park, New York

March 1996 - October 1996   Senior Research Fellow
            Division of Hematology/Oncology

Children's Hospital of Pittsburgh
Pittsburgh, Pennsylvania

September 1993 - February 1996   Post-Doctoral Fellow
Lab of Molecular Pharmacology
Division of Cancer Treatment
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

October 1992 - September 1993   Research Associate
Department of Chemistry
University of Leicester
Leicester, United Kingdom

April 1989 - November 1989   Visiting Fellow
Radiation Biology Unit
Paul Sherrer Institute
Villington, Switzerland

April 1988 - March 1989   Visiting Fellow
Institute of Radiation Biology
Zurich University
Zurich, Switzerland

August 1984 - March 1988   Research Assistant
Department of Health Physics
Institutes of Atomic Energy
Beijing, P. R. China

AWARDS AND HONORS:

(1) Career Development awards, Army Medical Research and Materiel Command (1999)
(2) LIJMC Faculty Research Pool Award, LIJMC, New York (1999)
(3) Awards of the 33rd Annual Academic Competition, LIJMC, New York (1998)
(4) LIJMC Faculty Research Award, LIJMC, New York (1997)
(5) Outstanding Awards of Department of Radiation Oncology, LIJMC, New York (1997)
(6) Awards of the 32nd Annual Academic Competition, LIJMC, New York (1997)
(7) NIH Research Fellowship, National Institutes of Health, Maryland (1996)
(8) NIH Fogarty Research Awards, National Institutes of Health, Maryland (1993 - 1996)
(9) Awards for Highest Student Standing, Department of Chemistry, University of Leicester, United Kingdom (1992)
(10) British Awards for Overseas Research Students (ORS), United Kingdom (1990-1992)
(11) National Awards of Scientific and Technical Progression, P. R. China (1988)
(12) Outstanding Young Scientist Awards of the Institutes of Atomic Energy, Beijing, P. R.

China (1986 - 1987)

BIBLIOGRAPHY: (Total: 54 articles)

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2. Andres J, Fan S, Turkel G, Twu R, Wang J, Yuan R, Goldberg ID and Rosen E. Regulation of BRCA1 and BRCA2 by DNA-damaging agents. Oncogene, 16: 2229-2241, 1998.

3. Fan S, Wang J, Yuan R, Andres J, Goldberg ID and Rosen E. Scatter factor protects epithelial and carcinoma cells against apoptosis induced by DNA damaging-agents. Oncogene, 17: 131-141, 1998.

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7. Rosen E, Fan S, Rockwell S and Goldberg ID. The molecular and cellular basis of radiosensitivity: Implications for understanding how normal tissue and tumors response to therapeutic radiation. Cancer Investigation, 17: 56-72, 1999.

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17. Meng Q, Qi M, Chen DZ, Yuan R, Goldberg ID, Rosen EM, Auborn K and Fan S. Suppression of breast cancer invasion and migration by indole-3-carbinol: Associated with up-regulation of BRCA1 and E-cadherin/catenin complexes. Journal of Molecular Medicine, 78: 155-165, 2000.

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Function Role of the BRCA1 LXCXE Motif in Regulation of RB Family Protein Expression And Cellular Chemosensitivity, but not RB Protein Binding

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ABSTRACT

The tumor suppressor activity of the BRCA1 gene product is due, in part, to functional interactions with other tumor suppressors, including p53 and the retinoblastoma (RB) protein. RB binding sites on BRCA1 were identified in the C-terminal BRCT domain (Yarden and Brody, *PNAS USA* 96: 4983-4988, 1999) and in the N-terminus (aa 304-394) (Aprelikova et al. *PNAS USA* 96: 11866-11871, 1999). The N-terminal site has a consensus RB binding motif, LXCXE (aa 358-362), but the role of this motif in RB binding and BRCA1 functional activity is unclear. We report that the interaction of RB with the N-terminal region of BRCA1 does <u>not</u> require the LXCXE motif, nor does it require an intact A/B binding pocket of RB. BRCA1 can also interact with the RB-related proteins p107 and p130. Expression of wild-type BRCA1 (wtBRCA1) caused down-regulation of expression of RB, p107, p130, and other proteins (eg., p300), associated with increased sensitivity to DNA-damaging agents; while while ectopic Rb expression caused up-regulation of BRCA1 expression. In contrast, expression of a full-length BRCA1 with an LXCXE inactivating mutation (LXCXE → RXRXH) failed to down-regulate RB, blocked the down-regulation of RB by wtBRCA1, induced chemoresistance, and abrogated the ability of BRCA1 to suppress tumor growth suppression of DU-145 prostate cancer cells. wtBRCA1-induced chemosensitivity was partially reversed by expression of either Rb or p300 and fully reversed by co-expression of Rb plus p300. Our findings suggest that: 1) some biologic functions of BRCA1 are mediated through an LXCXE site within the N-terminal RB binding region; and 2) BRCA1 and RB may reciprocally regulate each other's expression.

INTRODUCTION

BRCA1 gene mutations confer an increased risk for breast, ovarian, and prostatic cancers (1-3). The BRCA1 gene encodes an 1863 amino acid (aa), 220 kDa nuclear protein with an N-terminal RING domain that interacts with various cell cycle regulatory proteins (4) and a C-terminal transcriptional activation domain (TAD) (5) (see Fig. 1). Over-expression of BRCA1 inhibits cell growth and renders cells more susceptible to apoptosis (6-8); while decreased BRCA1 expression stimulates proliferation and is found in sporadic breast cancers (9). BRCA1 participates in the regulation of cell cycle, apoptosis, and DNA repair pathways (7-12). Breast cancers from patients with BRCA1 mutations have 2-3 fold more chromosomal aberrations than sporadic cancers (13), suggesting a role for BRCA1 as a "caretaker" gene in ensuring the maintenance of genomic integrity. We showed that BRCA1 inhibits the transcriptional activity of the estrogen receptor (ER-α), suggesting a potential role in breast cancer suppression by regulating estrogen-stimulated mammary cell growth (14).

Recent studies suggest that some of these BRCA1 functions may be mediated by functional interactions with two other tumor suppressor proteins: p53 and the retinoblasoma protein (RB). BRCA1 binds to p53 via two sites, one in the C-terminal minimal TAD (aa 1760-1863) and the other located N-terminally (aa 224-500) (15-17). The BRCA1:p53 interaction results in increased sequence-specific transcriptional activity of p53, suggesting that BRCA1 may function as a co-activator for p53. The C-terminus of BRCA1 also interacts with RB, two RB-associated proteins (RbAp46 and RbAp48), and histone deacetylases (HDAC-1 and HDAC-2) (18).

A second, more N-terminal RB-binding on BRCA1 was also identified (aa 304-394) (19). Furthermore, the ability of BRCA1 to induce cell cycle arrest at the G1/S border was significantly reduced in Rb (-/-) mouse embryo fibroblasts, as compared with the corresponding Rb (+/+) and Rb (+/-) cells (19). These findings suggest that some of the functional activities of BRCA1 may be mediated through a BRCA1:RB protein interaction. The N-terminal RB binding region of BRCA1

contains a consensus RB family protein binding motif, LXCXE (aa 358-362). However, the role of this [358]LXCXE motif of BRCA1 in mediating the BRCA1:RB physical interaction or its functional consequences is not known.

In this report, we demonstrate that the LXCXE motif embedded inside the N-terminal RB-binding region of BRCA1 is <u>not</u> necessary for binding of BRCA1 to RB, nor is an intact A/B binding pocket of RB required for binding. However, this motif is required for the functional consequences of the BRCA1:RB interaction, including the down-regulation of RB and the RB-related proteins (p107 and p130) and the induction of cellular chemosensitivity to DNA-damaging agents.

MATERIALS AND METHODS

BRCA1 and RB family expression vectors. *BRCA1.* The wild-type BRCA1 expression plasmid (wtBRCA1) was created by cloning the BRCA1 cDNA into the mammalian expression vector pcDNA3 (Invitrogen Corporation, Carlsbad, CA) using artificially engineered 5' Hind III and 3' Not I sites. Mutant BRCA1 and truncated BRCA1 expression vectors were created by modification of the wtBRCA1 cDNA in the pcDNA3 vector (see Fig. 1). The BRCA1 cDNAs utilized in this study are illustrated schematically in Fig. 1. *RB family.* The following expression vectors were used to express RB family proteins: pSG5-Rb, pCMV-p107, and pcDNA3-p130 (provided by Dr. Richard Pestell, Department of Medicine and Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York) (20). The p300 expression vector (pcDNA3-p300) was also provided by Dr. Richard Pestell.

GST-RB vectors for GST capture assays. The following vectors were utilized to express the ABC domains of GST-RB fusion proteins: pGEX2T-Rb (aa 379-928), pGEX2T-Rb 379-928 Δ exon 21 (missing aa 703-737), and pGEX2T-Rb 379-928 Δ exon 22 (missing aa 738-775) (21). These three vectors were provided by Dr. W. G. Kaelin, Jr. (Dana-Farber Cancer Institute, Boston, MA). The vectors pGEX2T-p107 (aa 252-936) (22) and pGEX3X-p130 (aa 322-1139) (23), which were utilized to express GST fusion proteins of the ABC domains of RB family proteins p107 and p130, respectively, were provided by Dr. Douglas Cress (H. Lee Moffitt Cancer Center and Research Institute, University of South Florida).

Sources of Reagents. The DNA-damaging agents adriamycin (ADR) (doxorubicin hydrochloride) and camptothecin (CPT) were purchased from the Sigma Chemical Company (St. Louis, MO). The primary antibodies used for Western blotting and their sources are described below under Western blotting. MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] was also

obtained from Sigma. G418 (geneticin) was obtained from Boehringer Mannheim. The cell permeable inhibitors of protein degradation used in this study were as follows: proteasomal degradation inhibitor (MG-132), caspase-3 inhibitor I, and caspase-6 inhibitor II. Each inhibitor was obtained from the Calbiochem-Novabiochem Corporation (La Jolla, CA)..

Tetracycline (TCN)-regulated BRCA1 expression system. The TCN-regulated expression system was created using the pTet-Off system (Clonetech Laboratories, Inc., Palo Alto, CA) (24). This system has two major components: 1) the regulator plasmid pTet-Off, which expresses a fusion of the Tet repressor (TetR) and VP16 activation domain of herpes simplex virus under control of the strong immediate early CMV promoter; and 2) the response plasmid pTRE, which contains the Tet-responsive element (TRE) (7 copies of 42 bp Tet operator) upstream of the minimal immediate early CMV promoter and a multicloning site (MCS).

DU-145 human prostate cancer cell clones were established by two separate stable transfections, the first with pTet-Off and the second with pTRE containing the full-length wtBRCA1 cDNA cloned into the MCS (pTRE-wtBRCA1). Double stable Tet-Off cell clones (designated DU-145/Tet-Off/BRCA1) were screened and chosen for BRCA1 expression induced by removal of TCN, by semi-quantitative RT-PCR analysis and by Western blotting (see below).

Cell lines, cell culture, and transfection. DU-145 human prostate cancer cells, MCF-7 human breast cancer cells, and other human cancer cell lines were originally obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 5% v/v (DU-145) or 10% v/v (MCF-7) fetal calf serum, L-glutamine (5 mM), non-essential amino acids (5 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (all obtained from BioWhittaker (Walkersville, MD) (8). Cell lines were incubated in a humidified atmosphere of 95% air and 5% CO_2 and were subcultured weekly, using trypsin.

For stable transfections, cells in 100 mm plastic Petri dishes at about 30-40% of confluence were incubated overnight with 5 µg of plasmid DNA, using Lipofectin (GIBCO Life Technologies, Rockville, MD), according to the manufacturer's instructions. Cells were then selected in G418 (0.5 mg/ml). The G418-resistant colonies were isolated using cloning rings, expanded, and screened for BRCA1 expression by Western blot and RT-PCR assays. Clones that stably over-expressed BRCA1 were frozen in liquid nitrogen.

For transient transfection assays, subconfluent proliferating cells were incubated overnight with 10 µg of plasmid DNA per 100 mm dish, in the presence of Lipofectin, and then washed to remove the Lipofectin and the excess plasmid DNA.

Semi-quantitative reverse transcriptase-polymerase chaim reaction (RT-PCR) analysis. The mRNA expression of the wild-type and mutant BRCA1 transgenes was verified by semi-quantitative RT-PCR assays, as described before by us (8). The BRCA1 primer sequences (5' → 3') and the locations within the cDNA sequence [GenBank accession number U15595 (1)] were as follows: sense TTG CGG GAG GAA AAT GGG TAG TTA; and antisense GAA GTA GTA AGT GGG AAC CGT GT (bp 5239 to 5523, 285-bp product). The Rb primer sequences (5' → 3') were as follows: sense TGC ACG AGT TGA CCT AGA TGA G; and antisense TCT TTG AGC AAC ATG GGA GG (bp 453 to 849, 397-bp product). The primers for the control gene, ß-actin, were: sense TTG TTA CCA ACT GGG GAC GAT A, antisense TCG TCC TTC TAG TTC TAG (bp 265 to 1028, 764-bp).

***In vitro* cell growth kinetics.** To assess *in vitro* cell proliferation, subconfluent proliferating cultures of two clones of each clonal type were harvested using trypsin. The cells were inoculated into six-well dishes at 3×10^4 cells per well in 5.0 ml of complete growth medium (DMEM plus 5% fetal calf serum) on Day 0. For each clone tested, duplicate wells were counted by hemacytometer on Days 1-8. The duplicate cell counts agreed to within ± 5% of the mean values.

***In vivo* tumor growth.** For each cell clone type tested, subconfluent proliferating cells were harvested; and a cell suspension containing 3×10^6 cells in 0.15 ml of a 50:50 mixture of DMEM plus Matrigel (Collaborative Research) was prepared. Four to six week old male athymic nude mice were injected subcutaneously with 3×10^6 cells per injection, two injections per animal (on the right and left sides), 10 animals per cell clone, and two cell clones per clonal type (= 20 injections per clonal type). Tumor volumes were measured twice per week by determining three mutually perpendicular diameters using calipers. Since the two individual clones of each type gave a very similar distribution of tumor sizes, these data were pooled for analysis of tumor sizes.

The volumes of the individual tumors were calculated according to the formula V (in mm^3) = $(\pi/6)$ $d_1 d_2 d_3$, where d_1, d_2, and d_3 represent the three perpendicular tumor diameters in mm. The mean tumor volume was calculated by averaging the tumor volumes corresponding to all of the injections that yielded tumors. Animals were sacrificed when the mean tumor diameters of the control (neo) cells reached about 15 mm. Statistical comparisons of the tumor growth rates of different clonal types were made using the two-tailed Student's t-test.

Drug treatments. Subconfluent proliferating cells were exposed to the indicated dosage of drug: DNA topoisomerase IIα inhibitor adriamycin (ADR), topoisomerase I inhibitor camptothecin (CPT), or sham treatment (negative control) for either 2 or 24 hr in complete culture medium. Cells were then washed to remove the drug and post-incubated in fresh drug-free culture medium for 24 hr for apoptosis assays and 48 hr for MTT cell viability assays.

MTT assay of cell viability. MTT assays are based on the ability of viable cells to convert MTT, a soluble tetrazolium salt (thioazyl blue) into an insoluble formazan precipitate, which is quantitated by spectrophotometry following solubilization in dimethyl sulfoxide (8,25). Briefly, subconfluent proliferating cells in 96-well dishes were treated with cytotoxic drug in standard growth medium, washed vigorously to remove the drug, and then post-incubated for 48 hr in fresh drug-free culture

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medium. At this time, the cells were solubilized and absorbance readings were taken using a Dynatech 96-well spectrophotometer. The amount of MTT dye reduction was calculated based on the difference between absorbance at 570 nm and at 630. Cell viability was expressed as the amount of dye reduction relative to that of untreated control cells. Ten replicate wells were tested per assay condition, and each experiment was repeated at least twice. Representative experiments are shown.

Apoptosis Assays. Exponentially growing cells in 100 mm plastic Petri dishes were treated ± drug in standard growth medium; washed twice to remove the drug; post-incubated in fresh drug-free medium for 24 hr; and counted. The samples were normalized by cell number (500,000-750,000 cells); and the low molecular weight apoptotic DNA was extracted, as described before (8,26). The DNA was electrophoresed through 1.2% formaldehyde-agarose gels containing 0.1 mg/ml of ethidium bromide; and the gels were photographed under ultraviolet illumination.

Immunoprecipitation (IP). Subconfluent proliferating cells in 150 cm^2 dishes were harvested, and nuclear extracts were prepared, as described earlier (27). Each IP was carried out using 6 µg of antibody or antibody combination and 1000 µg of nuclear extract protein. Precipitated proteins were collected using protein G beads, washed, eluted in boiling Laemmli sample buffer, and subjected to Western blotting. The BRCA1 IP antibody was as follows: combination of Ab-1 (MS110, Cat. #OP92) + Ab-2 (MS13, Cat. #OP93) + Ab-3 (SG11, Cat. #OP94), mouse monoclonals, Oncogene Research Products/Calbiochem (Cambridge, MA). The control IP antibodies were: normal mouse IgG or an irrelevant monoclonal (23C2) raised against hepatocyte growth factor/scatter factor (28).

Western blotting. Western blotting assays were performed as described earlier (8). Equal aliquots of protein extract (50 µg per lane) were electrophoresed on SDS-polyacrylamide gradient gels, transferred to nitrocellulose membranes (Millipore, Bedford, MA), and blotted as described earlier (8). The appropriate secondary antibodies (see below) were used at a dilution of 1:3000. Blotted

proteins were visualized using the enhanced chemiluminescence system (Amersham Life Sciences, Arlington Heights, IL), with colored markers (Bio-Rad Laboratories, Hercules, CA) as molecular size standards. The protein bands were quantitated by densitometry; and the values were expressed relative to the 43 kDa α-actin band as a control for loading and transfer.

The following primary antibodies for Western blotting were obtained from Santa Cruz Biotechnology, Inc.: BRCA1 (C-20, rabbit polyclonal IgG, 1:200 dilution), RB (C-15-G, goat polyclonal IgG, 1:500 dilution; or IF8, mouse monoclonal IgG$_1$, 1:200 dilution), p107 (SD9, mouse monoclonal IgG$_1$, 1:150 dilution), p130 (C-20, rabbit polyclonal IgG, 1:200 dilution), and α-actin (I-19, goat polyclonal IgG, 1:500 dilution). The antibodies against the three family proteins do <u>not</u> cross-react with each other. The sources and dilutions for all other antibodies (p300, BRCA2, Mdm-2, p21$^{Wafl/Cip1}$, Bcl-2, and Bax) were the same as described in an earlier publication (8).

Glutathione-S-transferase (GST) capture assays. GST capture assays were performed essentially as described earlier (29). ^{35}S-methionine-labeled proteins were prepared by *in vitro* transcription and translation, using the T7 promoter of the pcDNA3 vector. The GST fusion proteins were generated from cDNAs cloned into the GST vector (p-GEX), expressed in E. coli, and purified by affinity chromatography. *In vitro* translated labeled proteins were incubated with either GST alone (negative control) or GST fusion proteins for 4 hr at 4°C, recovered using GSH agarose beads, eluted in boiling Laemmli sample buffer, and analyzed by SDS-PAGE autoradiography. To confirm their expression, the GST fusion proteins were visualized by Western blotting, using an anti-GST mouse monoclonal antibody (B-14, Santa Cruz, 1:10,000 dilution).

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RESULTS

BRCA1:RB protein interaction does <u>not</u> require LXCXE (aa 358-362). We used the GST capture assay (29) to investigate the physical interaction between the BRCA1 and RB. The BRCA1 cDNAs tested in this study are illustrated in Fig. 1; and the ability of a GST-RB fusion protein containing the ABC domains of the wild-type RB protein (see Fig. 2a) to pull down different BRCA1 proteins is shown in Fig. 2b. GST-RB captured wtBRCA1 (aa 1-1863), Δ BamH1 (aa 1-1313), and Δ Kpn1 (aa 1-771) but did <u>not</u> capture Δ EcoR1 (aa 1-302). As a negative control, GST alone failed to capture any BRCA1 proteins. Further studies revealed that BRCA1 Δ Kpn1 and Δ BamH1 were pulled down by two GST-RB mutants defective in the A/B binding pocket: RB Δ Exon 21 and RB Δ Exon 22 (Fig. 2c). However, Δ EcoR1 (1-302) was <u>not</u> captured by any of these RB proteins. The RB Δ Exon 21 mutation corresponds to the RB mutation found in DU-145 cells (30).

Both wtBRCA1 and a full-length BRCA1 with the mutation ^{358}LXCXE → ^{358}RXRXH were captured by GST-RB, RB Δ Exon 21, and RB Δ Exon 22 (Fig. 2d). Somewhat surprisingly, two truncated forms of BRCA1, Δ Kpn1 (1-771) and Δ Msc1 (aa 1-440) with the mutation LXCXE → RXRXH were pulled down by each RB protein (Fig.2d). These findings suggest that there is an RB-binding site within aa 303-440 of BRCA1, but that neither the LXCXE site of BRCA1 (aa 358-362) nor an intact A/B binding pocket of RB is essential for the BRCA1:RB physical interaction.

As was observed for RB, the full-length wtBRCA1 protein was pulled down by GST fusion proteins containing the ABC domains of p107 and p130, but not by GST alone (Fig. 2e). Consistent with the findings for RB, GST-p107 and GST-p130 also captured the full-length BRCA1 protein containing the mutation LXCXE → RXRXH as well as a truncated BRCA1 protein containing only aa 1-771 (BRCA1 Δ Kpn1). However, in side-by-side comparisons, it appears that pull-down of BRCA1 by GST-p107 or GST-p130 was less than that of GST-RB. These findings suggest that p107 and p130 can also interact with the N-terminal region of BRCA1, and the LXCXE motif of BRCA1 is <u>not</u> required for the interaction of BRCA1 with p107 or p130.

BRCA1:RB interaction *in vivo*. Consistent with previous reports (18,19), we detected an *in vivo* interaction of BRCA1 and RB by IP (immunoprecipitation)-Western blotting. RB was detected in BRCA1 IPs of DU-145 prostate cancer cells and MCF-7 breast cancer cells; while control IPs with non-immune IgG or an irrelevant monoclonal yielded no RB band (illustrated in Fig. 3a). BRCA1 was detected in RB IPs in some but not all experiments (data not shown). The inability to consistently detect BRCA1 in RB IPs may be a technical problem related to disruption of the BRCA1:RB complex by the RB antibodies (C-15-G or IF8), since these antibodies effectively precipitated RB and since the *in vivo* BRCA1:RB interaction is well-documented by others (18,19).

Since the RB gene in DU-145 cells has an in-frame deletion of exon 21 (corresponding to RB Δ Exon 21 in the GST capture assays), an intact A/B binding pocket does <u>not</u> appear to be necessary for the BRCA1:RB interaction *in vitro* or *in vivo*. Next, we performed IP-Western blots of DU-145 cells stably transfected with wtBRCA1 or BRCA1-RXRXH. Consistent with the *in vitro* binding assays, RB was detected in the BRCA1 IP of BRCA1-RXRXH clones. But surprisingly, there was little or no RB in the BRCA1 IP of the DU-145 wtBRCA1 clone (Fig. 3b); and a similar result was obtained using wtBRCA1 and BRCA1-RXRXH clones of MCF-7 cells, which have wild-type RB (see Fig. 3c).

Consistent with a prior study (19), it was primarily the hypo-phosphorylated form of RB (lower M_r) that associated with BRCA1 (compare RB in MCF-7 lysates vs that in BRCA1 IPs in Figs. 3a, 3b, and 3c. Finally, the BRCA1:RB association was also demonstrated in other untransfected human breast cancer (T47D, MDA-MB-231, MDA-MB-453) and untransfected human prostate cancer (TsuPr-1, LnCAP) cell lines (data not shown).

BRCA1 down-regulates the expression of RB and RB-related proteins. To facilitate studies of BRCA1 functional activity in DU-145 cells, we developed cell lines expressing the wtBRCA1 gene under the control of a tetracycline (TCN)-regulated promoter system. Cells were subjected to two

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transfections using the Tet-Off system (Clonetech): 1) plasmid pTet-Off; and 2) plasmid pTRE-wtBRCA1 (containing the Tet operator and wtBRCA1 cDNA). Clones were screened for BRCA1 expression when TCN (2 μg/ml) was removed from the medium.

The BRCA1 mRNA and protein levels were increased at 24 hr or more after the removal of TCN from DU-145/Tet-Off/BRCA1 clones, but not from control (DU-145/Tet-Off/Neo) cell clones (Fig. 4a). Removal of TCN from the medium caused a quantitative reduction in RB mRNA and protein levels at 24, 48, and 72 hr in DU-145/Tet-Off/BRCA1 cells (see Figs. 4b and 4c).

In parallel to the reduction in RB, the levels of the RB-related proteins p107 and p130 were also significantly decreased at 24-72 hr after removal of TCN from the culture medium (Fig. 4c). Similarly, stably transfected wtBRCA1 clones of DU-145 cells exhibited reduced levels of RB, p107, and p130, as compared with control (neo) cell clones (Fig. 4d). Treatment of the wtBRCA1 stable DU-145 clone with inhibitors of proteasomal degradation (MG-132), caspase-3, or caspase-6 caused little or no change in the levels of RB, p107, or p130 (Fig. 4d). Finally, MCF-7 cell clones transfected with the wtBRCA1 cDNA also showed reduced levels of the RB, p107, and p130 proteins, as compared with control (neo) cell clones (illustrated in Fig. 4e).

BRCA1-RXRXH fails to down-regulate RB and reverses wtBRCA1-mediated down-regulation. We utilized a DU-145/Tet-Off/BRCA1 cell line to determine the effect of the BRCA1-RXRXH transgene on the expression of RB and the RB-related proteins. Cells were transfected overnight with BRCA1-RXRXH and incubated in the presence (+) or absence (-) of TCN for different times before harvesting for Western blot analysis. As expected, the removal of TCN from the medium caused down-regulation of RB, p107, and p130 protein levels, as compared with cells incubated +TCN (Fig. 5). Cells transfected with BRCA1-RXRXH and incubated +TCN failed to exhibit a decrease in the protein levels of RB, p107, or p130, suggesting that the mutation LXCXE $\rightarrow$ RXRXH abrogates the ability of BRCA1 to down-regulate RB family genes.

Furthermore, cells transfected with BRCA1-RXRXH and incubated -TCN also failed to down-regulate RB and only partially down-regulated the p107 and p130 proteins, suggesting that

when wtBRCA1 and BRCA1-RXRXH are co-expressed, BRCA1-RXRXH fully or partially blocks the ability of wtBRCA1 to induce the down-regulation of RB family protein levels. Alternatively stated, the BRCA1-RXRXH gene "rescues" the wtBRCA1-mediated down-regulation of RB expression.

Reciprocal regulation of BRCA1 levels by RB. Since BRCA1 causes decreased expression of RB, we performed the reciprocal study to determine if RB could regulate the levels of BRCA1. As demonstrated using our standard semi-quantitative RT-PCR assay (8), DU-145 and MCF-7 cells transfected with a full-length RB expression vector showed significantly higher levels of BRCA1 mRNA at 24 hr than cells transfected with the empty vector, as a negative control (Fig. 6). The implications of these findings are described below (see Discussion).

BRCA1-RXRXH confers a phenotype opposite to wtBRCA1 in DU-145 cells. To determine if inactivation of the LXCXE site alters the functional activity of the BRCA1 protein, we compared the phenotype of stably transfected BRCA1-RXRXH vs wtBRCA1 (positive control for BRCA1 function) vs empty vector (neo) (negative control) DU-145 cell clones. The total BRCA1 mRNA and protein levels were similar in RXRXH vs wtBRCA1 clones; and both clonal types had higher BRCA1 mRNA and protein levels than the control (neo and parental) cells (Fig. 7a).

Major differences were observed in the chemosensitivity of these clonal types. Thus, wtBRCA1 cell clones were more sensitive than neo to the DNA topoisomerase IIα inhibitor adriamycin (ADR) and to the topoisomerase I inhibitor camptothecin (CPT). However, BRCA1-RXRXH cell clones were more resistant to ADR than the neo clones (Fig. 7b). Agarose gel electrophoresis revealed that the BRCA1-RXRXH clones were more resistant and the wtBRCA1 clones were more sensitive than control (neo) cells to ADR- or CPT-induced apoptosis (Fig. 7c). Similar results were obtained using MCF-7 cell clones (Fig. 7d), demonstrating that the BRCA1-RXRXH gene also induces chemoresistance in a breast cancer cell line with wild-type RB.

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There were only minor differences in the *in vitro* growth kinetics of the different DU-145 clonal types. The population doubling times (T_d) were: 20 hr (BRCA1-RXRXH), 25 hr (wtBRCA1), and 22 hr (neo) (see Fig. 8a). To determine if the LXCXE → RXRXH mutation influences *in vivo* tumor growth, we compared the growth of DU-145 cell clones as tumors in the subcutaneous tissue of male nude mice. The wtBRCA1 clonal tumors showed a significantly decreased growth rate as compared with control (neo) clones ($P < 0.001$) (Fig. 8b). In contrast, the BRCA1-RXRXH clonal tumors continued to grow rapidly, and their growth pattern was indistinguishable from that of the control (neo) tumors ($P > 0.1$).

RXRXH phenotype demonstrated by transient transfection assays. We observed the RXRXH-mediated chemoresistance as well as RXRXH-mediated reversal of wtBRCA1-induced chemosensitivity in transient transfection assays using a DU-145/Tet-Off/BRCA1 clone (Fig. 9). In these experiments, transient transfection of BRCA1-RXRXH caused a decrease in sensitivity to ADR in cells expressing baseline endogenous levels of BRCA1 (+TCN conditions); and BRCA1-RXRXH overcame the chemosensitization induced by wtBRCA1 expression following the remoival of TCN (-TCN conditions). These results parallel the studies of RB family protein regulation, in which BRCA1-RXRXH failed to down-regulate these proteins and inhibited the ability of wtBRCA1 to cause down-regulation of the protein levels.

Regulatory protein expression in DU-145 BRCA1-RXRXH vs wtBRCA1 cell clones. We reported that DU-145 cell clones stably transfected with wtBRCA1 showed a set of alterations in the levels of various cellular regulatory proteins, as compared with control (parental and neo-transfected) cells: ↑ BRCA2, ↓ p300, ↓ Mdm-2, ↓ p21$^{Wafl/Cip1}$, ↓ Bcl-2, and ↓ Bax (8). A variety of other cellular proteins were not altered by wtBRCA1 [eg., Bcl-X_L, PCNA (proliferating cell nuclear antigen), CBP (CREB-binding protein), α-actin]. To determine if BRCA1-RXRXH induces alterations in these proteins, we compared the protein levels in three BRCA1-RXRXH clones vs three control (neo) clones (negative control), and one wtBRCA1 clone (positive control).

Western blots and densitometry analyses of protein levels are shown in Figs. 10a and 10b, respectively. As expected based on our previous study, the wtBRCA1 clone showed increased levels of BRCA1 and BRCA2 and decreased levels of p300, Mdm-2, p21$^{Wafl/Cip1}$, Bcl-2, and Bax. The BRCA1-RXRXH clones showed increased levels of BRCA1 protein, consistent with the expression of the BRCA1-RXRXH protein. However, the levels of BRCA2 in BRCA1-RXRXH clones were not markedly increased, and the levels of the other proteins were not significantly decreased, relative to neo clones. In fact, the levels of Mdm-2, which induces cell cycle progression and inhibits p53, were 2.6-fold higher in BRCA1-RXRXH clones than in neo clones. Thus, the LXCXE → RXRXH mutation abrogates the ability of BRCA1 to induce alterations in regulatory protein levels.

Reversal of wtBRCA1-mediated chemosensitivity by co-expression of RB and p300 cDNAs. In the studies described above, the wtBRCA1-induced down-regulation of RB family and other regulatory proteins appeared to correlate with an increased sensitivity to the DNA-damaging agent ADR. We used the TCN-regulated wtBRCA1 expression system in DU-145 cells to determine if co-expression of RB family genes along with wtBRCA1 could block the BRCA1-induced chemosensitivity. DU-145/Tet-Off/BRCA1 cells were transiently transfected with expression vectors for RB, p107, p130, or empty pcDNA3 vector and then tested for ADR-induced cytotoxicity, in the presence (+) or absence (-) of TCN. The results are shown in Fig. 11.

In the +TCN cells, exposure to ADR (15 μM x 2 hr) resulted in 70-75% cell viability after 48 hr, in untransfected or empty vector transfected control cells. The expression of BRCA1 by removal of TCN (-TCN) caused a reduction of cell viability to 17-20% in the control or vector transfected cells. However, transfectiuon of each of the three Rb family genes or all three genes together caused modest but significant increases in cell viability in the -TCN cells, to the range of 29-41%. Transfection of the p300 expression vector caused an even larger increase in cell viability, to about 60%; while the co-transfection of RB plus p300 restored the survival of ADR-treated cells to over 90%, even under -TCN conditions. Thus, it appears that in the presence of ectopically expressed (Rb + p300), wtBRCA1 expression no longer induces chemosensitivity to ADR in DU-145 cells.

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DISCUSSION

We showed that BRCA1 can bind to the RB protein through an N-terminal binding site located between aa 302 and 440. This finding is consistent with a previous report demonstrating an RB-binding site between aa 304 and 394 (19). However, we further showed that the consensus RB-binding motif LXCXE (aa 358-362) is not required for the BRCA1:RB interaction *in vitro* or *in vivo*. Thus, the mutation LXCXE → RXRXH did not abrogate the binding of RB to BRCA1, although our studies do not indicate whether this mutation alters the quantitative binding affinity. Furthermore, mutations that disrupt the configuration of the A/B binding pocket of RB (RB Δ Exon 21 and Δ Exon 22) - the domain that contacts the LXCXE sites of various RB-binding proteins (31,32) - did not abrogate the BRCA1:RB interaction. Taken together, these findings suggest that the BRCA1: RB interaction does not involve a typical LXCXE:pocket domain interaction.

Although not all LXCXE-containing proteins interact with RB, the fact that the BRCA1 LXCXE (aa 358-362) is embedded inside of an RB-binding region suggests that it may play some functional role related to the RB:BRCA1 interaction, even though it is not required for the binding. A clue to this functional role is the finding that stable or transient expression of a wtBRCA1 gene caused down-regulation of RB, p107, and p130 expression; while expression of a full-length BRCA1 gene with the mutation LXCXE → RXRXH did not affect the expression of these proteins. Along with the down-regulation of RB family proteins, cells expressing wtBRCA1 were more sensitive to cytotoxicity and apoptosis induced by the DNA-damaging agents ADR and CPT; while cells expressing BRCA1-RXRXH were less sensitive to these agents. Similarly, wtBRCA1 enhanced and BRCA1-RXRXH reduced the cellular sensitivity to ionizing radiation (unpublished data).

Interestingly, transient expression of wild-type RB in DU-145 (which contain a single mutant RB allele) caused the up-regulation of BRCA1 mRNA and protein levels. These findings suggest a reciprocal regulatory mechanism in which the reduction of RB levels by BRCA1 feeds back to cause

down-regulation of BRCA1, until a stable equilibrium point is reached. Our findings contrast with a recent study in which it was found that a segment of human BRCA1 gene promoter (-567) linked to a luciferase reporter was activated by E2F-1 and inhibited by over-expression of Rb, through a consensus E2F-1 binding site (33). The apparent Rb-mediated repression of BRCA1 might be due to the use of a small segment of the BRCA1 promoter that excludes a more 5' positive regulatory site.

The latter study was performed using the human cervical cancer cell line C33A (33). We had previously reported that, unlike various breast and prostate cancer cell lines, wtBRCA1 produced only very weak inhibition of estrogen receptor transcriptional activity in this cell line (14). Interestingly, C33A cells lack BRG-1, a component of the SWI/SNF transcriptional complex that has been found to co-operate with RB in mediating transcriptional repression and cell growth suppression (34). The role of BRG-1 in mediating the BRCA1 regulation of RB expression and vice versa remains to be determined.

If the hypothesis of a reciprocal regulatory mechanism involving BRCA1 and RB is correct, then gene mutations that disrupt the ability of BRCA1 to regulate RB and vice versa, could also disrupt the ability of these proteins to regulate the cell cycle and to mediate tumor suppression. This idea is consistent with the finding of a reduced ability of wild-type BRCA1 to cause a G1/S cell cycle block in Rb (-/-) mouse embryo fibroblasts (MEFs), as compared with Rb (+/-) and Rb (+/+) cells (19). It is also interesting to note that Brca1 (-/-) MEFs show a severe deficit in cell proliferation, while no such defect is exhibited in Rb (-/-) MEFs. If the above hypothesis is correct, Brca1 (-/-) cells should have very high levels of Rb, consistent with growth suppression, while Rb (-/-) cells should have lower levels of Brca1, consistent with their ability to proliferate *in vitro* and *in vivo*.

Our findings suggest that the LXCXE motif in the N-terminal RB-binding domain of BRCA1 participates in the regulation of RB family protein expression as well as the regulation of cellular chemosensitivity and apoptosis. Since a variety of studies have implicated RB in the regulation of

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apoptosis (35-37), the ability of BRCA1 to induce chemosensitivity and increased susceptibility to apoptosis (7,8,38) may be due, in part, to its ability to down-regulate RB expression through a mechanism that requires the LXCXE motif. One mechanism by which RB regulates apoptosis is by inhibition of apoptosis caused by the cell cycle-regulated transcription factor E2F-1 (39-41). E2F-1 causes apoptosis by a mechanism dependent upon its DNA-binding domain but not its transcriptional activation domain (TAD); and over-expression of RB blocked the E2F-1 induced apoptosis (41). Although the TAD of E2F-1 was not required for apoptosis induction, the RB-binding domain, which is located within the TAD, was required for the inhibition of E2F-1 mediated apoptosis by RB (41).

The mechanism of E2F-1 induced apoptosis is not entirely clear, but the study cited above suggests that relief of active repression of genes targetted by the E2F-1 transcription factor may be involved. The binding of RB to the E2F-1 TAD and the recruitment of histone deacetylases (HDACs) by the A/B binding pocket of RB provides a mechanism for active repression of genes containing E2F-1 binding sites (42,43). Presumably, the DNA-binding domain of E2F-1 is sufficient to induce apoptosis by displacing E2F-1:RB:HDAC repression complexes from the E2F-1 sites of target genes, thus stimulating transcription from these genes (41). One of these E2F-1 target genes is INK4a/p14^{ARF} (44). The p14^{ARF} protein and its murine homolog p19^{ARF} interact with Mdm-2 and block Mdm-2 mediated degradation of p53, thus resulting in stabilization of p53 (45,46). In fact, it was recently demonstrated that BRCA1, which functions as a co-activator for p53 (15-17), can stabilize the p53 protein through a mechanism that requires p14^{ARF} (47).

The above mechanism might explain the ability of the wtBRCA1 gene to induce chemosensitivity in cells with wild-type RB and p53 (eg., MCF-7). However, it does not explain the BRCA1-induced chemosensitivity of DU-145 cells (ref. 8 and this study), since these cells have mutations of both Rb (an in-frame deletion of exon 21) and p53 (a double point mutation of the sequence-specific DNA-binding domain) (30,48). Thus, if the A/B pocket-defective mutant RB of

DU-145 cells is unable to inhibit E2F-1, the down-regulation of RB induced by wtBRCA1 would not be expected to cause chemosensitivity.

Several explanations might account for the ability of wtBRCA1 to enhance chemosensitivity and susceptibility to apoptosis in DU-145 cells: 1) the pocket-defective mutant RB in these cells can modulate chemosensitivity by a mechanism independent of E2F-1; 2) one of the RB-related proteins p107 or p130, both of which are expressed in DU-145 cells, can substitute for this function of RB; and 3) wtBRCA1-mediated chemosensitivity could be due, in part, to mechanisms independent of the RB protein family. The ability of BRCA1 to induce down-regulation of p107 and p130 in DU-145 cells is consistent with the second explanation, as is the observation that BRCA1 can also interact with the p107 and p130 proteins.

The mechanism by which wtBRCA1 (but not BRCA1-RXRXH) causes down-regulation of RB and the RB-related proteins is not clear at this time. However, wtBRCA1 expression caused a significant reduction of RB mRNA as well as protein levels; and the protein levels were not restored by treatment with inhibitors of the ubiquitin-proteasome pathway or with inhibitors of caspases-3 and -6. These findings suggest that the BRCA1 regulation of RB occurs primarily at the mRNA level and raise the possibility that the RB gene may be a target for transcriptional repression by BRCA1.

Recent studies suggest a role for BRCA1 in transcriptional repression. For example, BRCA1 inhibited the transcriptional activity of the estrogen receptor (14). And the C-terminal region of BRCA1 was found to interact with transcriptional co-repressors, such as HDACs and the C-terminal interacting protein (CtIP), which recruits the co-repressor CtBP (C-terminal binding protein) (18,49). These findings are consistent with the observation that BRCA1 down-regulates other gene products at the mRNA level, such as the transcriptional co-activator p300 (8). The ability of wtBRCA1 to inhibit p300 expression was also abrogated by the LXCXE $\rightarrow$ RXRXH mutation.

The chemosensitivity induced by expression of wtBRCA1 was partially dependent upon both RB and p300, since co-expression of RB plus p300 blocked wtBRCA1-mediated chemosensitivity. Like RB, BRCA1 was found to interact directly with CBP/p300 (50). Binding of CBP was mediated by BRCA1 fragments containing aa 1-303 and aa 1314-1863, but not by a fragment containing the N-terminal RB protein binding site (aa 303-772). However, the role of the BRCA:CBP/p300 interaction in modulating chemosensitivity remains to be determined.

Interestingly, the expression of BRCA1-RXRXH not only abrogated the ability of BRCA1 to induce chemosensitivity, but also conferred chemoresistance and resistance to apoptosis induction in several different cell types. While the failure of BRCA1-RXRXH to induce down-regulation of RB and RB-related proteins could explain a failure to induce chemosensitivity, it would not explain why cell lines that express the BRCA1-RXRXH protein are actually more chemoresistant than control (parental or vector-transfected) cell lines. The chemoresistance of these cells might be explained by inhibition of the endogenous wild-type BRCA1 by BRCA1-RXRXH. The latter possibility is suggested by the finding that transient expression of a BRCA1-RXRXH genes blocks the chemosensitization of DU-145/Tet-Off/BRCA1 cells induced by removal of TCN from the medium.

The ability of BRCA1-RXRXH to block the chemosensitization of DU-145 cells induced by the over-expression of wtBRCA1 correlated with its ability to block the wtBRCA1-mediated down-regulation of RB and the RB-related proteins (p107 and p130) in the same cell line under the same conditions. The BRCA1-RXRXH protein might act to sequester other BRCA1-interacting proteins, thus disrupting specific protein:protein interactions required for the BRCA1-mediated down-regulation of the RB gene family and the BRCA1-mediated chemosensitivity.

In conclusion, we found that the LXCXE motif within the N-terminal RB-binding domain of BRCA1 is not necessary for the physical interaction of BRCA1 and RB but is required for two potential functional consequences of that interaction: 1) the down-regulation of expression of RB

family proteins; and 2) the BRCA1-induced chemosensitivity and susceptibility to apoptosis induction by DNA-damaging agents. Thus, the [358]LXCXE motif of BRCA1 is a functionally important site in mediating some of the tumor suppressor functions of BRCA1 and RB.

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FIGURE LEGENDS

Fig. 1. Schematic diagrams of BRCA1 expression vectors utilized in this study. The diagrams in Fig. 1. show the proteins encoded by the BRCA1 cDNAs: wild-type BRCA1 (wtBRCA1), BRCA1-RXRXH, Δ BamH1, Δ Kpn1, Δ Kpn1-RXRXH, Δ Msc1, Δ Msc1-RXRXH, and Δ EcoR1. All BRCA1 cDNAs are contained in the expression vector pcDNA3. *Abbreviations*: BRCT, BRCA1 C-terminal homology domain; LXCXE, consensus retinoblastoma protein binding motif; NLS1, primary nuclear localization signal; RING, ring finger (zinc finger-like) domain; TAD, minimal transcriptional activation domain.

Fig. 2. *In vitro* BRCA1:RB protein interaction does <u>not</u> require ^{358}LXCXE. a. Schematic diagrams of the GST-RB fusion proteins utilized in this study. The GST-p107 and GST-p130 constructs are described in the Materials and Methods section. b. Ability of GST-wild type RB to pull down different truncated BRCA1 proteins. c. BRCA1 Δ BamH1 (aa 1-1313) and BRCA1 Δ Kpn1 (1-771) but <u>not</u> Δ EcoR1 (aa 1-302) are pulled down by GST-wild-type RB as well as two A/B pocket domain mutants (GST-RB Δ Exon 21 and GST-RB Δ Exon 22). d. The LXCXE motif of full-length or C-terminally truncated BRCA1 proteins is <u>not</u> required for pull down by wild-type or A/B pocket domain mutant forms of GST-RB. e. GST fusion proteins containing the ABC domains of RB, p107, and p130 all pull down IVT wtBRCA1 (lanes marked a), BRCA1-RXRXH (lanes marked b), and BRCA1 Δ Kpn1 (lanes marked c).

Fig. 3. BRCA1:RB protein association *in vivo* in DU-145 and MCF-7 cells. a. RB is detected in BRCA1 immunoprecipitates (IPs) from untransfected DU-145 (prostate) and MCF-7 (breast) cancer cell lines by Western blotting. In contrast, control IPs using normal mouse IgG or an irrelevant mouse monoclonal (23C2) yielded no BRCA1 or RB protein bands. b. RB is detected in BRCA1 IPs from DU-145 cell clones stably transfected with empty vector (neo) or BRCA1-RXRXH but <u>not</u> in clones transfected with wtBRCA1. c. RB is detected in MCF-7 cell clones transfected with empty vector (neo) or BRCA1-RXRXH but <u>not</u> wtBRCA1.

Fig. 4. BRCA1 down-regulates RB family protein expression in DU-145 and MCF-7 cells. a. BRCA1 mRNA and protein levels are increased following the removal of tetracycline (TCN) from the culture medium from DU-145/Tet-Off/BRCA1 but <u>not</u> control (DU-145/Tet-Off/Neo) clones. Semi-quantitative RT-PCR and Western blotting of BRCA1 were performed as described before (8). b and c. Up-regulation of BRCA1 by removal of TCN from a DU-145/Tet-Off/BRCA1 clone causes a decrease in RB mRNA (b) and protein (c) levels. The levels of the RB-related proteins p107 and p130 are also decreased. d. Stably transfected DU-145 wtBRCA1 cell clones also exhibit reduced RB family (RB, p107, and p130) protein levels, as compared with control (neo) cell clones; and the levels of these proteins were not restored by treatment for 24 hr with inhibitors of the proteasomal degradation pathway (MG-132, 10 μM), caspase-3 (Casp-3 I, 25 μM), or caspase-6 (Casp-6 I, 25 μM). e. MCF-7 breast cancer cells stably expressing wtBRCA1 show decreased RB family protein levels, as compared with control (neo) cells.

Fig. 5. BRCA1-RXRXH blocks the down-regulation of RB expression mediated by wtBRCA1. Subconfluent proliferating DU-145/Tet-Off/BRCA1 cells were transfected overnight ± BRCA1-RXRXH (15 μg plasmid DNA per 100 mm dish) and then incubated in the presence (+) or absence (-) of TCN (2 μg/ml) for the indicated times. Cells were then harvested and subjected to Western blot analysis (50 μg cell protein per lane) to detect the RB, p107, p130, and α-actin (control) proteins.

Fig. 6. Expression of the Rb gene causes up-regulation of BRCA1 mRNA expression in DU-145 and MCF-7 cells. Subconfluent proliferating cultures of DU-145 and MCF-7 cells were transfected overnight with a wild-type Rb expression vector (+) or the empty vector (-) (10 μg plasmid DNA per 100 mm dish), washed, and incubated for 48 hr in fresh culture medium. Cultures were harvested to assess the levels of BRCA1 and Rb mRNAs by semi-quantitative RT-PCR analysis.

Fig. 7. BRCA1-RXRXH confers a phenotype characterized by chemoresistance and resistance to apoptosis in DU-145 and MCF-7 cells. a. Stably transfected DU-145 BRCA1-RXRXH and wtBRCA1 clones show increased BRCA1 protein levels, as compared with control (neo) clones. b. DU-145 wtBRCA1 clones are more sensitive, while BRCA1-RXRXH clones are less sensitive to the DNA-damaging agent adriamycin (ADR) than control (neo) cell clones. Cell viability was measured 72 hr after exposure to ADR, using the MTT assay, as described before (8,25). Values are means ± SEMs from three clones of each type. For each clone, ten replicate wells were tested. c. DU-145 wtBRCA1 clones are more susceptible, while

BRCA1-RXRXH clones are less susceptible to apoptosis induction by ADR, as demonstrated by DNA ladder assays. Low molecular weight DNA was isolated 24 hr after exposure to adriamycin; and the DNA was electrophoresed through a 1.5% agarose gel containing ethidium bromide, as described earlier (8,26). d. Assays of cell viability and apoptosis revealed that wtBRCA1 clones of MCF-7 cells are more sensitive and BRCA1-RXRXH clones of MCF-7 cells are more resistant than control (neo) cell clones.

Fig. 8. Effect of LXCXE → RXRXH mutation on cell proliferation and on tumor growth in DU-145.
Stably transfected DU-145 BRCA1-RXRXH cell clones grew at a similar rate to neo clones and slightly faster than wtBRCA1 clones *in vitro* (panel a). Cell counts are means ± ranges of two clones of each clonal type. For each individual clone, duplicate wells were counted. The population doubling times (T_d) were: neo, 22 hr; BRCA1-RXRXH, 20 hr; and wtBRCA1, 25 hr. Subcutaneous tumor growth rates of wtBRCA1 vs BRCA1-RXRXH vs control (neo) clones were determined as described in the Materials and Methods section (panel b). For almost all time points, the wtBRCA1 tumors grew significantly more slowly than the neo tumors ($P < 0.001$. two-tailed t-test); while the growth rate of BRCA1-RXRXH tumors was similar to that of neo tumors ($P > 0.1$).

Fig. 9. Transient RXRXH expression causes chemoresistance and reverses wtBRCA1-mediated chemosensitivity. Transient transfection of BRCA1-RXRXH but not pcDNA3 vector conferred increased survival after exposure to ADR in cells with baseline BRCA1 levels (+TCN). Over-expression of wtBRCA1 in the same cells (-TCN) conferred increased sensitivity to ADR. The wtBRCA1-induced sensitization to ADR was reversed by transfection of BRCA1-RXRXH but <u>not</u> empty pcDNA3 vector. Comparisons of cell viability of BRCA1-RXRXH transfected cells vs control (untransfected or pcDNA3 vector-transfected) cells yielded $P < 0.001$ for both +TCN and -TCN conditions.
Methodology. DU-145/Tet-Off/BRCA1 cells were transfected overnight with BRCA1-RXRXH, pcDNA3 vector, or vehicle only (15 µg per 100mm dish). Cells were washed and incubated in fresh medium for 3 hr. They were then harvested with trypsin, counted, seeded into 96-well dishes (3000 cells/well) without or with TCN (2 µg/ml), and allowed to attach and recover for 24 hr. Cells were incubated with ADR (15 µM x 2 hr), washed, post-incubated in drug-free medium for 48 hr, and assayed for MTT dye conversion. Cell viability is expressed relative to controls; and the values are means ± SEMs of ten replicate wells.

Fig. 10. BRCA1-RXRXH fails to induce the same protein alterations as wtBRCA1 in DU-145 cells. Subconfluent asynchronously proliferating clones of neo (N=3), BRCA1-RXRXH (N=3), and wtBRCA1 (N=1) DU-145 cell clones were harvested for extraction of total cell protein and Western blotting. Equal aliquots of total cell protein (50 μg per lane) were blotted to detect BRCA1, BRCA2, p300, Mdm-2, p21$^{Wafl/Cip1}$, Bcl-2, Bax, and α-actin (control) (panel a). Panel b shows the ratios of protein/actin determined by densitometric analysis of the protein bands in panel a. Values are mean ± SEMs for three clones each of neo and BRCA1-RXRXH and the corresponding values for the one wtBRCA1 cell clone.

Fig. 11. Reversal of BRCA1-mediated chemosensitivity by the co-expression of RB and p300. Transient expression of RB, p107, p130, or all three genes partially blocked the increased chemosensitivity to adriamycin (ADR) caused by the expression of wtBRCA1 following the removal of tetracycline (TCN) from the culture medium of subconfluent proliferating DU-145/Tet-Off/BRCA1 cells. Expression of a wild-type p300 gene partially but strongly blocked the chemosensitivity. However, co-expression of p300 plus RB restored the BRCA1-induced chemosensitivity essentially to the control (+TCN) levels.
Methodology. Subconfluent proliferating DU-145/Tet-Off/BRCA1 cells were transfected overnight with the indicated vector(s) (10 μg of plasmid DNA per 100 mm dish), washed, and allowed to recover by incubation in fresh culture medium for 3 hr. The cells were then harvested with trypsin, counted, seeded into 96-well dishes (3000 cells/well) without (-) or with (+) TCN, and allowed to attach and recover for 24 hr. Cells were incubated with ADR (15 μM x 2 hr), washed, post-incubated in drug-free culture medium for 48 hr, and assayed for MTT dye conversion. Cell viability is expressed relative to non-ADR treated control cells. Cell viability values are the means ± SEMs of ten replicate wells.
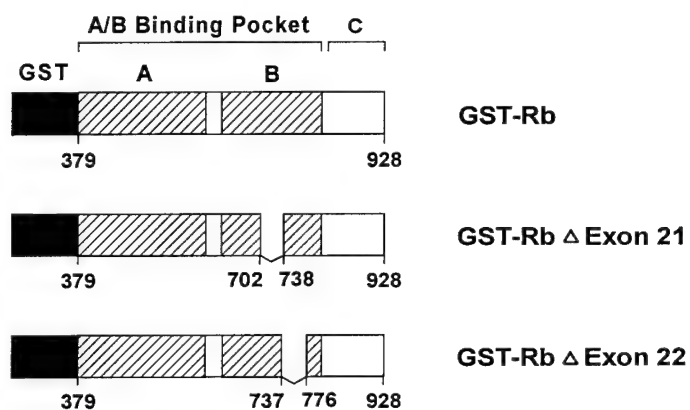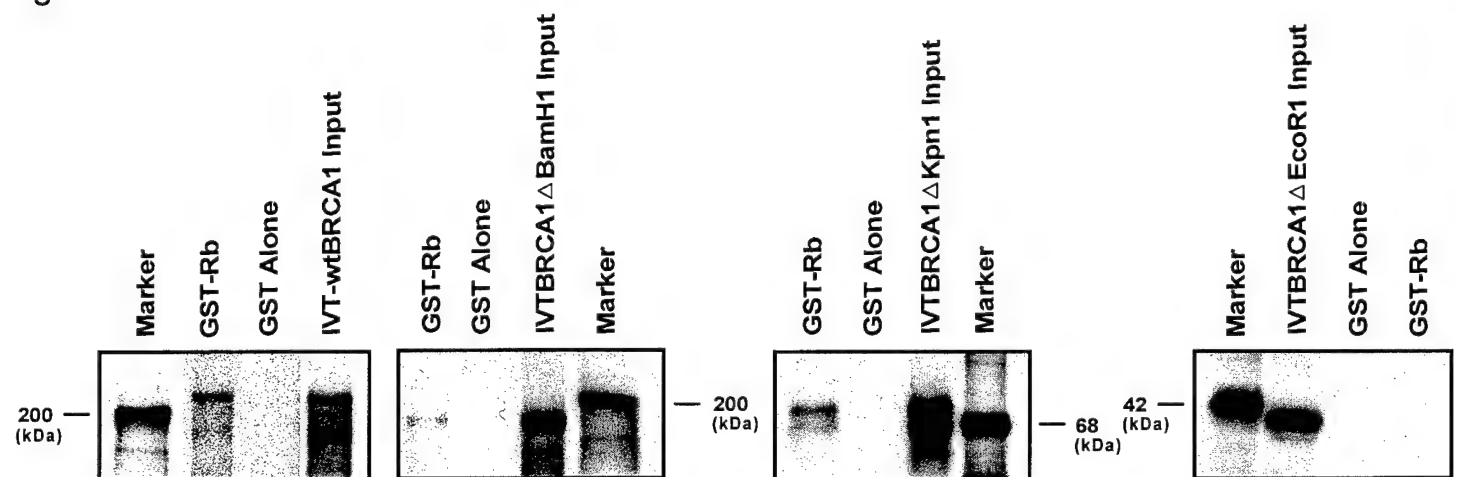
Fig. 1

Fig. 2a

GST-Rb
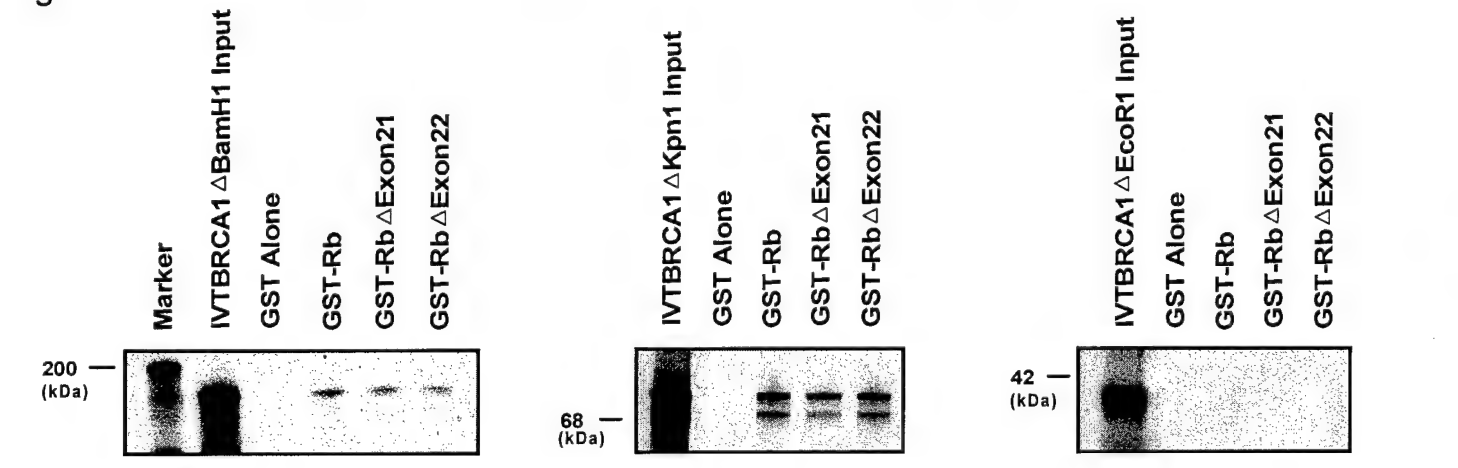379 — 928

GST-Rb △ Exon 21
379 — 702 — 738 — 928

GST-Rb △ Exon 22
379 — 737 — 776 — 928

A/B Binding Pocket: A, B, C
GST

Fig. 2b

Marker, GST-Rb, GST Alone, IVT-wtBRCA1 Input
GST-Rb, GST Alone, IVTBRCA1△BamH1 Input, Marker
GST-Rb, GST Alone, IVTBRCA1△Kpn1 Input, Marker
Marker, IVTBRCA1△EcoR1 Input, GST Alone, GST-Rb

200 (kDa)
200 (kDa)
68 (kDa)
42 (kDa)

Fig. 2c

Marker, IVTBRCA1△BamH1 Input, GST Alone, GST-Rb, GST-Rb△Exon21, GST-Rb△Exon22
IVTBRCA1△Kpn1 Input, GST Alone, GST-Rb, GST-Rb△Exon21, GST-Rb△Exon22
IVTBRCA1△EcoR1 Input, GST Alone, GST-Rb, GST-Rb△Exon21, GST-Rb△Exon22

200 (kDa)
68 (kDa)
42 (kDa)

Fig. 2d

Marker
IVTwtBRCA1 Input
GST Alone
GST-Rb
GST-Rb △ Exon21
GST-Rb △ Exon22

200 (kDa) —

Marker
IVTBRCA1-RXRXH Input
GST Alone
GST-Rb
GST-Rb △ Exon21
GST-Rb △ Exon22

68 (kDa) —

IVTBRCA1△Kpn1 Input
GST Alone
GST-Rb
GST-Rb △ Exon21
GST-Rb △ Exon22
IVTBRCA1△Kpn1-RXRXH Input
GST Alone
GST-Rb
GST-Rb △ Exon21
GST-Rb △ Exon22

68 (kDa) —

Marker
IVT △ Msc1 Input
GST Alone
GST-Rb
GST-Rb △ Exon21
GST-Rb △ Exon22

Marker
IVT △ Msc1-RXRXH Input
GST Alone
GST-Rb
GST-Rb △ Exon21
GST-Rb △ Exon22

Fig. 2e

126 (kDa) —
90 —

GST-Rb
GST-p107
GST-p130
GST Alone

Rb
p107
p130

208 (kDa) —
126 —

a b c

IVT wtBRCA1
IVTBRCA1-RXRXH
IVTBRCA1△Kpn1 Input

GST Alone

GST-Rb

GST-p107

GST-p130

Fig. 3a

BRCA1 IB (C-20)
209 —
125 —
RB IB (C-15)

MCF-7 Lysate

MCF-7 } BRCA1 IP
Du-145 } (Ab-1, 2, 3)

Mouse IgG } Control IP
23C2 } (MCF-7)

RB (105-125 kDa)

BRCA1 (220 kDa)

Fig. 3b

BRCA1 IB (C-20)
209 —
125 —
RB IB (C-15)

MCF-7 Lysate
Mouse IgG IP (Du-145)
MCF-7
Du-145/Neo-2
Du-145/wtBRCA1-6 } BRCA1 IP (Ab-1, 2, 3)
Du-145/RXRXH-32
Du-145/RXRXH-34

RB (105-125 kDa)

BRCA1 (220 kDa)

Fig. 3c

BRCA1 IB (C-20)
209 —
125 —
RB IB (C-15)

MCF-7/Neo-2 } Lysate
MCF-7/wtBRCA1-2
Normal Mouse IgG IP (MCF-7)
MCF-7/Neo-2
MCF-7/wtBRCA1-2 } BRCA1 IP (Ab-1, 2, 3)
MCF-7/BRCA1-RXRXH-2
MCF-7/BRCA1-RXRXH-13

RB (105-125 kDa)

BRCA1 (220 kDa)

Fig. 4d

Fig. 4e

Fig. 4a

Fig. 4b

Fig. 4c
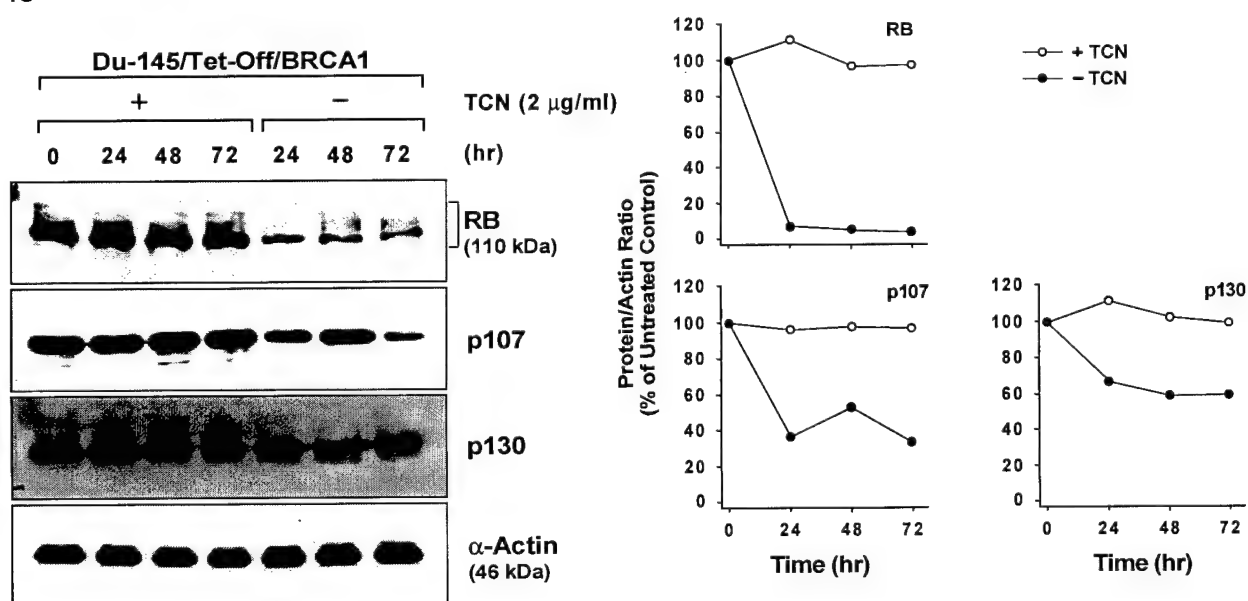
Fig. 5

Fig. 6

Fig. 7a

Fig. 7b

Fig. 7c

ADR
(15μM
x 24 hr)

CPT
(15μM
x 24 hr)

Fig. 7d

CPT
(15μM
x 24 hr)

Fig. 8b

Fig. 8a

Fig. 9

ADR 15 μM x 2 hr
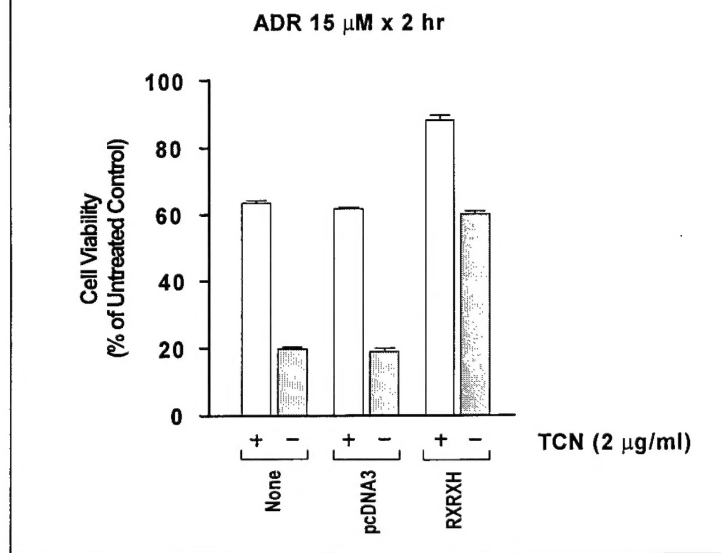
Cell Viability (% of Untreated Control)

TCN (2 μg/ml)

None   pcDNA3   RXRXH

Fig. 10b

Fig. 10a

Fig. 11